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Certification of the amount-of-substance fraction of HbA1c versus the sum of all Hb isoforms forming the glycated or non-glycated N-terminal hexapeptide of the β -chain in haemoglobin isolated from whole blood IRMM/IFCC-466

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REFERENCE MATERIALS

Certification of the amount-of-substance fraction of HbA1c versus the sum of all Hb isoforms forming the glycated or non-glycated N-terminal hexapeptide of the *β*-chain in haemoglobin isolated from whole blood

IRMM/IFCC-466

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SUMMARY

This report describes the preparation, homogeneity, stability and characterisation studies as well as the purity evaluation of highly purified HbA1c (IRMM/IFCC-466) from human blood. HbA1c is defined as the stable adduct from glucose and the N-terminal amino group of the β-chain of haemoglobin A0 that is beta-N-(1-deoxyfructos-1-yl) haemoglobin [1]. A description of the analytical methods used in each of these studies is included. All relevant data from the homogeneity and stability studies as well as those for the characterisation measurements are presented.

The certified value is:

	Certified value[1)] Amount-of-substance fraction [mmol/mol]	Uncertainty[2)] [mmol/mol]
HbA1c/(HbA1c + HbA0)[3)]	934	22

[1)] The certified value was calculated as the average of the results for the amount-of-substance fraction of HbA0 versus HbA0 plus HbA1c of three accepted datasets and converted into amount-of-substance fraction HbA1c (1000 mmol/mol – HbA0 mmol/mol). Measurements were carried out using the IFCC reference measurement procedure [2] and further confirmed by other methods. The certified value, is traceable to the SI.

[2)] The certified uncertainty is the expanded uncertainty estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM), ISO, 1995. It is expressed with a coverage factor (k = 2), corresponding to a level of confidence of about 95 %.

[3)] HbA1c is defined as the beta-N-(1-deoxyfructos-1-yl) haemoglobin. HbA0 is defined as the non-glycated haemoglobin.

The impurity consisting of HbA0 in IRMM/IFCC-466 was found to be 66 mmol/mol ± 22 mmol/mol (expressed as HbA0 per HbA1c plus HbA0).

The total haemoglobin mass fraction in the material was also determined and the corresponding value (26.2 ± 0.9 mg Hb/g of solution) is provided as additional material information.

The intended use of this certified reference material is the calibration of the IFCC reference measurement procedure and other analogous methods based on the quantification of the N-terminal hexapeptide that includes the stable glycation.

The commutability of the material with routine *in vitro* diagnostic devices has not been assessed. Thus, prior to be used as calibrant for routine *in vitro* diagnostic devices, the commutability of IRMM/IFCC-466 should be investigated by the user.

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GLOSSARY

2D-Gel	Two-dimensional gel electrophoresis
ANOVA	Analysis of variance
CEofix	Capillary Electrophoresis ofix HbA1c kit Analysis
CE/UV	Capillary electrophoresis with ultraviolet detection
CyanMetHb	Cyanmethaemoglobin
CRM	Certified Reference Material
CV	Coefficient of variation
EC-JRC	European Commission – Joint Research Centre
EDTA	Ethylenediaminotetraacetic acid
ESI/MS	Electrospray ionization mass spectrometry
F	Snedecor F
F_{crit}	Critical value of Snedecor F
FSQ Unit	Food Safety and Quality Unit of IRMM
Hb	Haemoglobin (total)
HbA0	Non-glycated haemoglobin
HbA1c	Glycated haemoglobin defined beta-N-(1-deoxyfructos-1-yl) haemoglobin
HPLC	High performance liquid chromatography
ICSH	International Council for Standardisation in Haematology
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
INSTAND, e.V.	Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e.V.
IRMM	Institute for Reference Materials and Measurements
k	Coverage factor
ℓ	Number of laboratories
LOD	Limit of detection
M_{HbA1c}	Molar mass of HbA1c
M_{HbA0}	Molar mass of HbA0
MES	2-Morpholinoethanesulfonic acid
MetHb	Methaemoglobin
$MS_{between}$	Mean of squares between groups
MS	Mean of squares
MS_{within}	Mean of squares within groups
m_R	Relative molecular mass
n	Number of replicates

N	Number of vials
ν	Degrees of freedom
p-value	Error probability
RM	Reference Material
RM Unit	Reference Materials Unit of IRMM
RSD	Relative standard deviation
s	Standard deviation
s_b	Standard deviation of the slope
s_{bb}	Standard deviation between bottles
s_{wb}	Standard deviation within bottles
SKB	Streekziekenhuis Koningin Beatrix
SS	Sum of squares
θ	Temperature (°C)
$t_{0.05,\nu}$	t of Student test at 95 % of confidence and ν degrees of freedom
TosohA1	Ion exchange chromatographic system from Bioscience Inc, for HbA1c.
TosohA2	Ion exchange chromatographic system from Bioscience Inc, for HbA0.
U	Expanded uncertainty
u_{bb}	Standard uncertainty component from homogeneity study
u^*_{bb}	Standard uncertainty of the maximum hidden between bottle heterogeneity
u_{char}	Standard uncertainty component from batch characterisation
U_{CRM}	Expanded uncertainty of the certified value expressed with a coverage factor k = 2, corresponding to a level of confidence of about 95 %
u_{lts}	Standard uncertainty component from long-term stability study
u_{sts}	Standard uncertainty component from short-term stability study
USM	Università degli Studi di Milano
UV-vis	Ultraviolet-visible

1. INTRODUCTION

Diabetes mellitus is a collection of disorders associated with hyperglycaemia, a chronic elevation of the concentration of glucose in the blood, as a result of insulin deficiency, insulin resistance or a combination of both. This condition affects more than 5 % of the world's population. The major medical and social problems are the chronic diabetic complications, which in the end may lead to blindness, renal failure and/or limb amputations. The risk for these complications strongly depends on the long-term glycaemic status of the patients. The closer the blood glucose concentrations are maintained within the normal intervals, the lower the risk of such complications. At present the diagnostic control for the estimation of this long-term glycaemic status is based on the determination of haemoglobin-glucose adducts (glycated haemoglobins) in blood, formed by non-enzymatic glycation of haemoglobin. The major form, about 80 %, of these glycated haemoglobins is haemoglobin A1c (HbA1c), resulting from the condensation of glucose with the N-terminal valine residue of each β-chain of haemoglobin and subsequent formation of a stable ketoamine. HbA1c has been defined as beta-N(1-deoxy) fructos-1-yl haemoglobin, while HbA0 is the non-glycated form of haemoglobin. Formation of glycated haemoglobin is essentially irreversible, and the blood level depends on both the life span of the red blood cells and the blood glucose level. The important role played by the determination of HbA1c vs HbA0 levels to monitor the evolution of patients with diabetes mellitus under treatment implies that the analysis of HbA1c is very frequently requested in laboratory medicine.

Despite the above-mentioned clinical importance of the HbA1c determination as a support in clinical diagnosis, it is known that the reliability and comparability of test results from the large variety of commercial methods available are unsatisfactory. Inter-laboratory comparisons have confirmed that the variation is too high and mainly due to systematic differences between methods. For these reasons, it is currently not possible to recommend scientifically founded decision limits as guidance for the treatment of diabetic patients. The methods are based on a variety of analytical principles such as charge differences (ion exchange chromatography in HPLC or mini-columns, electrophoresis), affinity binding of glycated haemoglobins (affinity chromatography) or the immunological behaviour of HbA1c (immunoassays).

These stated discrepancies in the HbA1c measurements led IFCC to establish a working group having the task to develop a reference system for the international standardisation of all HbA1c assays. The use of calibrators had previously demonstrated to decrease the inter-laboratory and inter-method dispersion of glycated haemoglobin results [2, 3, 4]. Therefore, among the priorities of this reference system was the production of certified primary reference materials as well as the establishment of a suitable reference method for the quantification of HbA1c *vs.* HbA0.

The IFCC Working Group on HbA1c Standardization has developed a reference measurement procedure. This method quantifies the cleaved N-terminal hexapeptide of the β-chain of haemoglobin. The glycated and non-glycated hexapeptides are first separated by HPLC and then quantified by mass spectrometry (HPLC-ESI/MS) or by UV detection after a second separation step by capillary electrophoresis (HPLC-CE/UV). Both detection systems give identical results when calibrated with mixtures of highly purified HbA0 and HbA1c. The performance of the reference measurement procedure was validated by a network of reference laboratories [5].

IRMM/IFCC-466 is a deep-frozen buffered solution containing HbA1c. It was prepared from whole blood obtained from diabetic volunteers and provided by Roche Diagnostic GmbH, Department for Biochemical Materials, Penzberg, Germany.

The major objectives of the project included the processing of the material, the evaluation of the purity of the candidate reference material and the subsequent characterisation and assessment of homogeneity and stability. IRMM/IFCC-466 is to be used in combination with IRMM/IFCC-467 for the calibration of the IFCC reference measurement procedure in the interval of clinical interest (0 to 150 mmol/mol for amount-of-substance fraction HbA1c in HbA0 plus HbA1c).

The intended use of this material (IRMM/IFCC-466), along with IRMM/IFCC-467 (HbA0, non-glycated haemoglobin in deep frozen buffered solution prepared from whole blood), as primary calibrants for the reference measurement procedure, required us to test for additional impurities not detected by the reference method. While the IFCC reference measurement procedure is highly selective for HbA1c and HbA0 since it specifically measures the N-terminal hexamer of the β-chain of haemoglobin ignoring the presence of other isoforms, the additional methods employed (Tosoh system, CEofix system, UV/Vis spectrometry and 2D gel electrophoresis) involve the evaluation of intact haemoglobin or its intact chains.

2. PARTICIPANTS

2.1 Processing and bottling of the certified reference material IRMM/IFCC-466

Roche Diagnostics GmbH, Department for Biochemical Materials, Penzberg, DE

2.2 Homogeneity and stability studies

Streekziekenhuis Koningin Beatrix (SKB), Winterswijk, NL

2.3 Characterisation measurements

Streekziekenhuis Koningin Beatrix (SKB), Winterswijk, NL

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2.4 Evaluation of additional impurities

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2.5 Evaluation and uncertainty estimation

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3. CERTIFICATION PROJECT TIME SCHEDULE

The timing of the project was as follows:

- Processing of the material: June 2002
- Shipment to IRMM: July 2002
- Characterisation study: April 2004
- Homogeneity study (evaluated with data from stability studies)
- Stability studies
    - Short-term: March 2004 – October 2004
    - Long-term 1 year: January 2003 – January 2004
    - Long-term 2 years: January 2003 – January 2005
- Testing for additional impurities: May 2006

4. PREPARATION OF THE MATERIAL

4.1 Description of the material

IRMM/IFCC-466 was prepared by Roche Diagnostics GmbH from whole blood obtained from diabetic volunteers.

The purification process for HbA1c was performed by multi-step liquid chromatography [8]. HbA0 and HbA1c were separated using cation exchange chromatography (SP Sephadex) and each of them further purified by sequential affinity chromatography (Mono S) and cation exchange chromatography (SP Sephadex).

The material consists of a deep frozen buffered solution containing HbA1c.

4.2 Bottling of samples

A total number of 100 vials, each containing 8 mg of HbA1c in 0.310 mL buffer solution (50 mmol/L MES, 10 mmol/L KCN, 2 mmol/L EDTA, pH 6.2), were produced and bottled at Roche Diagnostics GmbH in June 2002.

4.3 Dispatching of samples

Samples were dispatched to the laboratories performing the characterisation and stability studies. All samples were packed in special containers filled with dry ice and were dispatched within 24 hours to the laboratories.

5. MEASUREMENT PROCEDURES

Various analytical methods were used for the determinations of total Hb and HbA1c and/or HbA0. Quantification of HbA1c and HbA0 was carried out using the IFCC reference measurement procedure described in section 5.2 while the total haemoglobin mass fraction was determined by the use of the ICSH reference method (cyanmethaemoglobin method described in section 5.1).

The relatively small batch size of IRMM/IFCC-466 produced required a combined design of the homogeneity, stability and characterisation studies.

5.1 Method used for stability and homogeneity studies

The mass fraction of total haemoglobin in solution was determined according to the recommended ICSH reference method [6]. Total haemoglobin mass fraction is measured at 546 nm in the form of cyanmethaemoglobin. This is formed from the reaction between the Drabkin's reagent and the haemoglobin previously oxidised to methaemoglobin.

This approach was selected for the stability study based on the following assumptions: a) equal degradation of both HbA1c and HbA0 since they differ just in the glycation state and, b) equal degradation of total Hb and N-terminal hexapeptides of HbA0 and HbA1c, since the total amount of Hb in IRMM/IFCC-466 is considered to be comprised of HbA1c and a small amount of HbA0.

For the evaluation of the homogeneity study, it can be assumed that the same homogeneity will be found either for the whole molecule or for a peptide derived from the whole molecule since the material is a solution.

5.2 Methods used for characterisation

The characterisation of the material was performed using the IFCC reference measurement procedure [5]. After cleavage of haemoglobin into peptides, the N-terminal hexapeptide corresponding to the β-chain of haemoglobin is selected as final and unique target for the determination of both forms HbA0 and HbA1c, the latter defined as beta-N(1-deoxy) fructos-1-yl haemoglobin.

HPLC-ESI/MS and HPLC-CE/UV were used to determine the identity and amount-of-substance fraction of the isoform of HbA1c forming the glycated N-terminal hexapeptide of the β-chain versus HbA1c plus HbA0 and other isoforms forming the glycated or non-glycated hexapeptide of the β-chain in IRMM/IFCC-466. From now on in this report, the amount-of-substance fraction of the HbA1c isoform forming the glycated N-terminal hexapeptide of the β-chain versus the sum of the HbA1c isoform forming the glycated N-terminal hexapeptide of the β-chain and all haemoglobin isoforms including HbA0 forming the non-glycated N-terminal hexapeptide of the β-chain will be referred as the amount-of-substance fraction of HbA1c versus HbA1c plus HbA0.

The high specificity of the IFCC reference measurement procedure, measuring only the N-terminal hexapeptide of the β-chain, does not allow detecting the presence of other glycated isoforms of haemoglobin, or even the presence of other proteins in the material. These other

isoforms would interfere with the ICSH reference method when determining the total Hb mass fraction before preparing the calibration solutions by mixing IRMM/IFCC-466 with IRMM/IFCC-467 and would thus create a bias. In addition, neither the IFCC reference measurement procedure nor the ICSH reference method are able to provide information on the oxidation state of IRMM/IFCC-466.

For all reasons described above, it was decided to apply less specific techniques, described in section 5.3, to assess the presence of haemoglobin isoforms in IRMM/IFCC-466.

5.3 Methods used for testing additional impurities

a) 2D-gel electrophoresis was applied to IRMM/IFCC-466 and to IRMM/IFCC-466 spiked with IRMM/IFCC-467 to obtain an amount-of-substance fraction of 50 mmol/mol of HbA1c versus HbA1c plus HbA0 to asses the LOD of the method and to exclude the presence of HbA0 in the material.

b) The molecular mass of the α and β chains (in the glycated or non-glycated forms) was verified by analysing IRMM/IFCC-466 with two different LC-MS systems: a) reverse phase C4 column coupled to ion trap and b) reverse phase cyano-propyl column coupled to a triple quadrupole MS detector.

c) The presence of different haemoglobin isoforms was tested by applying commercially available systems: the capillary electrophoresis-based specific test for HbA1c (CEofix) and the ion-exchange HPLC-based Tosoh A1c and HPLC Tosoh A2 methods. These systems separate whole-tetrameric haemoglobins that are analysed by measuring their absorbance at 415 nm (wavelength characteristic of the Soret band of haemoglobin). The chromatographic Tosoh HbA1c system is optimised for the pH interval corresponding to the isoelectric point of HbA1c, while Tosoh HbA2 is optimised for the pH interval corresponding to the isoelectric point of the haemoglobin isoform HbA2 which is similar to that of HbA0. Therefore, Tosoh HbA1c shall be better suited for the evaluation of IRMM/IFCC-466 than Tosoh HbA2.

d) Finally, to evaluate whether HbA1c is undergoing oxidative processes, the absorption spectra in the interval of 400-750 nm of IRMM/IFCC-466 was compared to that obtained from a fresh haemolysate.

6. STABILITY STUDIES

The stability study started in January 2003 following an isochronous design [7]. The reference temperature for this study was –70 °C, at which no degradation of the analytes over a long period can be expected. Due to the limited batch size the number of vials submitted to the isochronous schemes, both for short- and long-term stability studies, was minimised.

The stability studies were based on the analysis of total Hb mass fraction by the ICSH reference method [6] and according to the assumptions described in section 5.1. That approach was found acceptable since no degradation of total haemoglobin implies no degradation of HbA1c and/or HbA0.

6.1 Short-term stability study

One sample was stored in the dark at –70 °C (reference temperature) and two samples were stored at –30 °C, one of them for 4 and the other for 8 weeks. Each sample was analysed in triplicate under repeatability conditions using the method described in section 5.1 and the average results are presented in Table 1a.

The resulting data were evaluated by performing a trend analysis for which results are illustrated in Table 1b.

Table 1a – Short-term stability of IRMM/IFCC-466 at –30 °C: Total Hb mass fraction expressed in mg/g of solution

mass fraction	storage time [week]		
	0	4	8
Total Hb [mg/g]	25.87± 0.06[1)]	25.97 ± 0.06[1)]	25.90 ± 0.10[1)]
[1)] average value ± *s* (*N* = 1, *n* = 3)			

Table 1b – Test for significance of the slope: slope (*b*), standard deviation (s_b) and b/s_b of short-term stability study for IRMM/IFCC-466

short-term stability study			*t*-test	
θ [°C]	*b* [(mg/g)/week]	s_b [(mg/g)/week]	$t = b/s_b$	$t_{0.05,7}$
–30 °C	0.0042	0.0084	0.50	2.365

The slope of the regression line (b) was tested for significance using the t-test and the data are shown in Table 1b. Since the t value obtained is smaller than t critical value ($t_{0.05,7}$), it is possible to conclude that the obtained slope is not significantly different from zero at 95 % confidence level.

Consequently, the samples are stable at –30 °C for at least 8 weeks. IRMM/IFCC-466 should be dispatched on dry ice and after reception, the vials should be kept at –70 °C in the dark (see section 6.2).

6.2 Long-term stability study

The long-term stability study was conducted over a period of 12 months following an isochronous design. Two samples were stored at –70 ºC, one for 6 and the other for 12 months. The reference temperature was about ─160 °C (storage above liquid N_2). The results obtained are illustrated in Table 2.

Table 2a – Long-term stability of IRMM/IFCC-466 at –70 ºC: Total Hb mass fraction expressed in mg/g of solution

mass fraction	storage time [month]		
	0	6	12
Total Hb [mg/g]	25.87 ± 0.06[1)]	25.90 ± 0.01[1)]	25.97 ± 0.12[1)]
[1)] average value ± s (N = 1, n = 3)			

Table 2b – Test for significance of the slope: slope (b), standard deviation (s_b) and b/s_b of short-term stability study for IRMM/IFCC-466

short-term stability study			t-test	
θ [°C]	b [(mg/g)/month]	s_b [(mg/g)/month]	$t = b/s_b$	$t_{0.05,7}$
–70 °C	0.0083	0.0047	1.76	2.365

Using a trend analysis (t-test) of the data, the slope was found not to be significantly different from zero at a confidence level of 95 %, indicating that the samples are stable at –70 °C for at least 12 months. The uncertainty contribution from stability to the total uncertainty budget is estimated according to the following expression [7]:

$$u_{lts} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} x$$

with x_i being the time points for the individual sample (i), x the shelf life and RSD the relative standard deviation of the results of the stability study.

A shelf-life of 49 months was calculated for the material, according to Pauwels *et al*. [7,8], with an expected relative standard uncertainty (u_{lts}) of 1 %. This value is included in the uncertainty budget.

7. HOMOGENEITY STUDY

Detailed evaluation of the values for total Hb mass fraction in solution (mg/g) from both, short-term and long-term stability studies of IRMM/IFCC-466, showed no significant trend of the data with time under the conditions tested. Therefore, it is feasible to assess the homogeneity of IRMM/IFCC-466 using the data of both the short-term and the long-term stability studies. The condition establishing a minimum number of units to be tested was met ($\sqrt[3]{N}$, N = 100). Since no differences were observed for the total haemoglobin mass fraction in solution between –30 °C and –70 °C, both sets of data were combined to estimate the uncertainty due to heterogeneity. Therefore, six vials analysed (N = 6) in triplicate (n = 3) were considered. The uncertainty contribution u_{bb} was estimated using one-way ANOVA and the results of the study are given in Table 3.

The evaluation of the homogeneity study is performed using the ICSH reference method under the assumptions described in section 5.1. Therefore, the uncertainty for the homogeneity, calculated from the evaluation of the total haemoglobin mass fraction, can be included in the uncertainty budget, U_{CRM}.

A reliable quantification of between unit heterogeneity is not possible due to the small number of replicates per unit. When evaluating the homogeneity of the material using ANOVA the maximum inhomogeneity that can be hidden by the method repeatability (u^*_{bb}) was calculated as follows [8] and included in Table 3:

$$u^*_{bb} = \sqrt{\frac{MS_{within}}{n}} \cdot \sqrt[4]{\frac{2}{N(n-1)}} \; ; \qquad s_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}}$$

Table 3 - ANOVA results for the homogeneity study of IRMM/IFCC-466 expressed as total Hb mass fraction in mg/g of solution.

Source of variation	SS	ν	MS	F	*p-value*	F_{crit}
Between bottle	0.031	5	0.006	1.12	0.401	3.106
Within bottle	0.067	12	0.006			
Total	0.098	17				

Using the results from the ANOVA (Table 3), the standard deviation between bottles (s_{bb}) and the standard uncertainty of the maximum hidden between-bottle heterogeneity (u^*_{bb}) were calculated according to the above described formulas.

- s_{bb}= 0.015
- u^*_{bb}= 0.028

Since u^*_{bb} has a bigger value than s_{bb}, u^*_{bb} is further taken into consideration as uncertainty component for the homogeneity.

Taking into account the average value obtained for total haemoglobin mass fraction in solution, 25.91 mg/g of solution, the relative standard uncertainty of the maximum hidden between-bottles heterogeneity can be calculated (u^*_{bb}/average = 0.11 %).

The measurements showed an acceptable level of inhomogeneity, when compared to the stability and characterisation contributions, all becoming part of the uncertainty budget (Table 6).

8. BATCH CHARACTERISATION

8.1 Evaluation of additional impurities

The absence of other proteins in IRMM/IFCC-466 was evaluated using 2D-gel electrophoresis. No contamination coming from other proteins was observed for the pH interval 3 to 10 and the molecular mass interval from 10 to 100 kDa on silver stained gels. Only 2D gel spots at molecular masses around 16 kDa, corresponding to the masses of α and β chains (glycated or non-glycated), were observed. A parallel experiment performed on a sample of IRMM/IFCC-466 (HbA1c) spiked with IRMM/IFCC-467 (HbA0) to a amount-of-substance fraction of 50 mmol/mol (HbA0/HbA1c+HbA0) showed no additional spots.

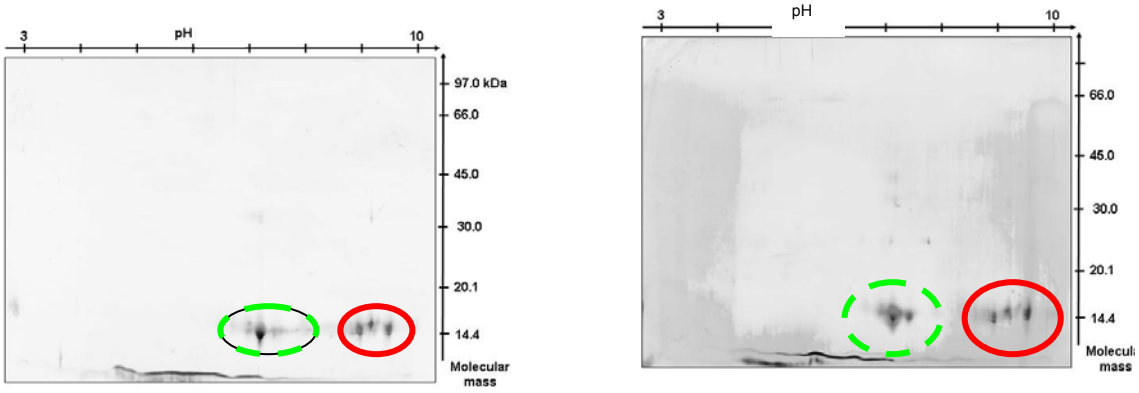

Figure 1. 2D-gel corresponding to IRMM/IFCC-466 (left) and IRMM/IFCC-466 (right) spiked with IRMM/IFCC-467 to obtain a amount-of-substance fraction of 50 mmol/mol of HbA1c versus HbA1c plus HbA0, the pH interval being 3-10 and the relative molecular mass interval 10-100 kDa. The α-chains are shown in red (continuous line) while the β-chains are shown in green (dashed line). The molecular mass of the α and β chains (in the glycated or non-glycated forms) of IRMM/IFCC-466 was verified by using two different LC-MS methods as described in section 5.3.

The observed molecular masses are 15126 daltons, 15867 daltons and 16029 daltons for the haemoglobin α-chain, non-glycated-β-chain and glycated-β-chain, respectively. The obtained results are in complete agreement with the theoretical molecular masses for the haemoglobin α-chain and β-chain (glycated and/or non-glycated).

The presence of different haemoglobin isoforms was tested by applying commercially available systems based on CE (CEofix) and HPLC (Tosoh) methodology as described in section 5.3. All chromatograms corresponding to these assays can be seen in Annex 1 (Figures I to IV) while the results are summarized in Table 4.

Table 4 – Characterisation of Hb isoforms in IRMM/IFCC-466 expressed as an area fraction (%) of all Hb isoforms absorbing at 415 nm, corresponding to the amount-of-substance fraction under the assumption that the molar extinction coefficients of all Hb isoforms are equivalent.

Component	Method [1)]		
	Tosoh HbA1c	Tosoh HbA2	CEofix A1c Analis kit
HbA1c	94.5 %	95.7 %	96.4 %
HbA0	1.4 – 2.0 %	1.3 %	1.1 %
HbX	2.9 – 3.4 %	1.5 %	2.5 %
[1)] CV: 1 % for the Tosoh HbA1c system, 3.8 % for the Tosoh HbA2 system and 2.0 % for the CEofix A1c analis kit when measuring IRMM/IFCC-466. Values are expressed as peak area percentage.			

In all cases, the estimated purity of HbA1c is higher than when using the IFCC reference measurement procedure as can be seen in section 8.2. HbX is eluting at the retention time corresponding to a labile HbA1c in the Tosoh HbA1c system and near the position of HbF (foetal haemoglobin isoform) in the Tosoh HbA2 and CEofix systems. However, the presence of such isoforms is not consistent with the 3-step purification procedure carried out for the preparation of IRMM/IFCC-466. The IFCC reference measurement procedure measures HbX as the non-glycated N-terminal hexapeptide as also derived from HbA0. When the area fraction of HbX is added to the area fraction of HbA0 the data are consistent with the results obtained with the IFCC reference measurement procedure and therefore it can be concluded that there are no other haemoglobin isoforms present in IRMM/IFCC-466 that would not be detected by the IFCC reference measurement procedure but with the ICSH reference method.

The peak corresponding to HbA1c is split when eluting from the chromatographic system Tosoh HbA1c (Figure I in Annex 1) but not when using the CEofix A1c system (Figure III in Annex 1). A plausible explanation for this observation is the existence of multiple oxidation stages for HbA1c in IRMM/IFCC-466. These multiple oxidation states are not distinguished by the CEofix system because the addition of KCN converts all of them into the uniform cyanmethaemoglobin derivatives. This was confirmed by adding KCN or oxidative conditions were applied to fresh haemolysate prior to using Tosoh chromatography.

This is corroborated by the absorption spectra, in the region of 400-750 nm for the group haeme, of IRMM/IFCC-466 and fresh haemolysate (Figure IV in Annex 1). The UV/vis absorption spectrum of IRMM/IFCC-466 clearly shows some degree of oxidation.

In summary, there is a good agreement among the values reported by the three commercially available systems. Both HbX and HbA0, impurities detected by the commercially available systems (CEofix, Tosoh A1c and Tosoh A2), will be detected as HbA0 when using the IFCC reference measurement procedure. The sum of the values reported for the impurities by the commercially available systems (expressed as the amount-of-substance fraction for HbA0 or HbX versus the sum of all detected isoforms, i.e. HbA1c, HbA0 and HbX) is somewhat lower than when using the IFCC reference measurement procedure. Therefore, these values obtained by the commercially available systems provide a solid base for the purity statement of the material using the IFCC reference measurement procedure since it is a more conservative estimation.

8.2 Characterisation

The goal of this study was to evaluate the amount-of-substance fraction of HbA1c versus HbA0 plus HbA1c and any isoform forming the glycated N-terminal hexapeptide in IRMM/IFCC-466. The batch characterisation was performed by three laboratories according to the IFCC reference measurement procedure that used as prescribed, either the HPLC-ESI/MS or HPLC-CE as detection system [5]. The amount-of-substance fraction of HbA0 in IRMM/IFCC-466 was evaluated by a standard addition protocol according to Finke *et al*. [9].

Laboratories 1 and 3 used the HPLC-ESI/MS detection system, while laboratory 2 used the HPLC-CE/UV system. Laboratory 1 performed a single measurement in duplicate on the pooled content of 5 vials (N = 1; n = 2). Laboratories 2 and 3 performed the digestion step on the individual vials. Laboratory 3 performed two measurements (N = 2 vials, 1 digestion each) in triplicate (n = 3) and laboratory 2 performed two measurements (N = 2 vials, 1 digestion each) in duplicate (n = 2).

All laboratories provided the results as the mass fraction HbA0 versus HbA1c plus HbA0, i.e. as mass fraction of HbA0 impurities in IRMM/IFCC-466. The results were converted to the amount-of-substance fraction by applying a conversion factor of 0.99 mol/mol (M_{HbA0} = 66910 g/mol, M_{HbA1c} = 67234g/mol). Finally, the amount-of-substance fraction of HbA1c can be calculated by subtracting the HbA0 amount-of-substance fraction from 1000 mmol/mol. The values, obtained according to the following formula, are summarised in Table 5.

$$HbA1c = 1000\,[mmol/mol] - \left(HbA0\,[mg/g] \times \frac{M_{HbA1c}[g/mol]}{M_{HbA0}[g/mol]} \right)$$

Table 5 – Results for the characterisation study of IRMM/IFCC-466 given as amount-of-substance fraction of HbA0 and HbA1c, respectively, versus the sum of HbA1c and HbA0.

	HbA0 Amount-of-substance fraction [mmol/mol][1,3)]	number of measurements [n]	HbA1c Amount-of-substance fraction [mmol/mol][2,3)]
Lab. 1 (HPLC-ESI/MS)	57.4 ± 1.4	2	942.6 ± 1.4
Lab. 2 (HPLC-CE/UV)	64.4 ± 0.4	2	935.6 ± 0.4
Lab. 3 (HPLC-ESI/MS)	76.2 ± 4.4	6	923.8 ± 4.4
Average	66.0	-	934.0

[1)] Values are expressed as the amount-of-substance fraction HbA0 ± s versus HbA1c plus HbA0.

[2)] Values corresponding to the amount-of-substance fraction HbA1c ± s versus HbA1c plus HbA0.

[3)] The values reported by the laboratories do not overlap because only information on the repeatability of the measurements is covered in the uncertainty stated.

9. UNCERTAINTY BUDGET AND ASSIGNED VALUE

The evaluation described hereafter is based on a concept reported by Pauwels *et al.* [10] and in the GUM [11] and uses available data discussed in the previous chapters.

9.1 Uncertainty evaluation

All three sets from the characterisation study (see Table 5) were taken into account for the calculation of the uncertainty u_{char}. Due to the low number of data sets, it was decided to determine the uncertainty budget for the batch characterisation according to Pauwels *et al.* [10].

Two uncertainty components were taken into consideration: a) the uncertainty that is exclusively laboratory-dependent [u_i] and b) the uncertainty component corresponding to the standard uncertainty of the average of the laboratory means [u_R]. The final equation for u_{char} is $u_{char} = \sqrt{u_i^2 + u_R^2}$. The components u_i and u_R can be calculated by using the following expressions:

$$u_i = \frac{\sqrt{\sum_{j=1}^{l} u_j^2}}{l} \quad ; \quad u_R = \frac{\bar{x}_{\max} - \bar{x}_{\min}}{\sqrt{3}}$$

Since laboratories provided results for the amount-of-substance fraction HbA0 vs HbA1c plus HbA0, the uncertainty estimation for the batch characterisation is calculated for HbA0 and directly applied in absolute values to HbA1c.

The residual component, u_R, of the uncertainty budget for the characterisation, u_{char}, was calculated applying a rectangular distribution because of the limited number of laboratories participating. Consequently, the uncertainty budget for the characterisation is shown in Table 6.

Table 6 – Uncertainty estimation for the batch characterisation of IRMM/IFCC-466

	CV [%] (for HbA0)	*u* [mmol/mol]
Lab 1	2.4	1.38[1)]
Lab 2	0.6	0.39[1)]
Lab 3	5.8	4.42[1)]
u_i	-	1.55
U_R	-	5.43
u_{char}[2)]	-	5.64

[1)] *u* is calculated as *s*

[2)] $u_{char} = \sqrt{u_i^2 + u_R^2}$

The standard uncertainty for the characterisation (u_{char}) is found to be 5.64 mmol/mol (expressed as mmol HbA1c per mol HbA1c plus HbA0).

9.1.1 Combined uncertainty

Based on the findings obtained in the stability and homogeneity studies as well as on the scattering of results in the interlaboratory batch characterisation, estimates for u_{bb} (homogeneity), u_{lts} (long-term stability) and u_{char} (batch characterisation) were combined and expanded according to the following equation:

$$U_{CRM} = 2 \cdot \sqrt{u*_{bb}^2 + u_{lts}^2 + u_{char}^2}$$

While the estimates for $u*_{bb}$ and u_{lts} were based on measurements made with the ICSH reference method evaluating the total haemoglobin mass fraction in solution, the calculations for u_{char} were performed using the IFCC reference measurement procedure evaluating just the N-terminal hexapeptide of the β-chain of the haemoglobin molecule. However, by taking the reasoning given in sections 6 and 7 into consideration, one can estimate the U_{CRM} just as described in the above equation.

9.1.2 Uncertainty budget

Based on the uncertainty contributions mentioned in sections 6.2, 7 and 9.1 the following uncertainty budget was established (Table 7):

Table 7 - Uncertainty budget for the certified value of IRMM/IFCC-466 expressed as mmol HbA1c per mol HbA1c plus HbA0.

uncertainties [mmol/mol]	
u^*_{bb} [1)]	1.03
u_{lts} [1)]	9.34
u_{char}	5.64
U_{CRM} (k = 2)	21.92
[1)] calculated from the values obtained for total Hb mass fraction in solution.	

The relative standard uncertainty of the maximum hidden between-bottles heterogeneity calculated using ANOVA, as described in section 6.2, is u^*_{bb}/average = 0.11 %. Therefore, the value for u_{bb} expressed in mmol/mol and included in the uncertainty budget is 1.03 mmol/mol.

As described in section 6.2, the expected relative standard uncertainty for the long-term stability (u_{lts}) was set to be 1 %. Therefore, the value expressed in mmol/mol included in the uncertainty budget is 9.34 mmol/mol.

9.2 Certified value

The amount-of-substance fraction certified in IRMM/IFCC-466 is **934 ± 22 mmol/mol** (expressed as mmol HbA1c per mol HbA1c plus HbA0).

9.3 Indicative value: total Hb mass fraction

The mass fraction of total Hb in solution for IRMM/IFCC-466 (glycated haemoglobin, HbA1c) was determined by using the ICSH reference method [6]. The colorimetric method was applied by two different laboratories. Laboratory 2 analysed five samples, those corresponding to the characterisation study and laboratory 1 analysed the samples corresponding to the stability study[1]. Therefore, the values from the stability studies were taken together with those of laboratory 2 for the evaluation of both the total haemoglobin mass fraction and its related uncertainty budget.

[1] The relatively large amount required for the HPLC-ESI/MS measurement did not allow the additional evaluation of the total Hb content on those samples.

Table 8a – Measurement of the total Hb mass fraction in solution for IRMM/IFCC-466 by using the ICSH reference method.

	total Hb [mg/g][1)]	*n*
Lab. 1 (STS + LTS)	25.91 ± 0.08	6
Lab. 2 (characterisation)	26.4 ± 0.55	5
Average (2 labs)	26.16	
[1)] Values are expressed as mass fraction of total Hb in solution ± *s*.		

The uncertainty was estimated following the approach described in section 9.1. As before, two uncertainty components were taken into consideration: a) the uncertainty that is exclusively laboratory-dependent [u_i] and b) the uncertainty component corresponding to the standard uncertainty of the average of the laboratory means (u_R). The results are shown in Table 8b.

Table 8b – Uncertainty associated to the total Hb mass fraction in solution estimated according to Pauwels *et al*. [9]

	u [mg/g]
Lab 1	0.05[1)]
Lab 2	0.55[1)]
u_i	0.28
u_R	0.14
u_{char}[2)]	0.31
[1)] *u* is calculated as the *s* [2)] $u_{char} = \sqrt{u_i^2 + u_R^2}$	

The residual component, u_R of the uncertainty budget for the characterisation, u_{char}, was estimated applying a rectangular distribution because of the limited number of laboratories participating (Table 8b). The uncertainty budget for the total haemoglobin mass fraction obtained according to section 9.3 is shown in Table 9. The uncertainty contributions for stability and homogeneity are those already described for the certified value since the method applied for those studies was the Drabkin's method. However, the value corresponding to u_{char} differs since both methods and analytes (first the N-terminal hexapeptide and now the total haemoglobin) are different.

Table 9 - Uncertainty budget for the total haemoglobin mass fraction in solution according to the ICSH method in IRMM/IFCC-466

Uncertainties	total Hb in solution in IRMM/IFCC-466 [mg/g]
u^*_{bb}	0. 03
u_{lts}	0.26
u_{char}	0.31
U_{CRM} (k = 2)	0.81

The standard uncertainty of the maximum hidden between-bottles heterogeneity calculated using ANOVA, as described in section 7, is u^*_{bb} = 0.03 mg/g.

As described in section 6.2, the expected relative standard uncertainty for the long-term stability (u_{lts}) was set to be 1 %. Therefore, the value expressed in mg/g included in the uncertainty budget is 0.26 mg/g.

The total haemoglobin mass fraction in solution calculated using the ICSH reference method and estimated to be 26.2 ± 0.9 mg Hb/g solution. The calculated shelf life, as described in section 6.2, is 49 months.

10. METROLOGICAL TRACEABILITY

The certified value, expressed as mmol HbA1c per mol HbA1c plus HbA0, is traceable to the SI. The assignment of the certified value was carried out using the IFCC reference measurement procedure [5] and further confirmed by other independent methods that tested the presence of impurities other than HbA0.

Initial separation of haemoglobin was accomplished using SP Sephadex. The identity of HbA1c and HbA0 was confirmed by the elution time on a Mono S HPLC column and the corresponding molecular masses were verified by two different LC-MS systems: a) reverse phase C4 column coupled to ion trap and b) reverse phase cyano-propyl column coupled to a triple quadrupole MS detector.

The purity of the material was assessed by HPLC-ESI/MS or HPLC/CE/UV applied on the digested N-terminal hexapeptide (glycated for HbA1c or non-glycated for HbA0) and excluding the presence of other Hb isoforms by using 2D gel electrophoresis, the ion-exchange HPLC-based Tosoh A1c and HPLC Tosoh A2 systems and the capillary electrophoresis-based specific test CEofix Analis. Finally, to evaluate whether HbA1c is undergoing oxidative processes, the absorption spectrum in the interval of 400-750 nm of IRMM/IFCC-466 was compared to that obtained from a fresh haemolysate.

11. INSTRUCTIONS FOR USE

11.1 Intended use

The intended use of this reference material is the calibration of the IFCC reference measurement procedure and analogous methods evaluating the stable glycated N-terminal hexapeptide of the β-chain of Hb. The material shall be used for the preparation of calibrants within the clinical relevant interval (25 – 110 mmol/mol of HbA1c versus HbA0 plus HbA1c).

Commutability of the material with routine *in vitro* diagnostic devices has not been assessed and if intended to be used as calibrant for routine *in vitro* diagnostic devices the user would have to assess commutability of IRMM/IFCC-466.

11.2 Transport and storage

The IRMM/IFCC-466 is supplied as a deep frozen buffer solution (50 mmol/L MES, 10 mmol/L KCN, 2 mmol/L EDTA at pH 6.2). Shipment will be carried out on dry ice. On receipt, the materials should be stored in the dark at -70 °C.

11.3 Minimum sample intake

The minimum sample intake is 20 µL.

REFERENCES

[1] K. Miedema. Standardization of HbA1c and optimal range of monitoring. Scand. J. Clin. Lab. Invest. Suppl., 240 (2005) 61-72

[2] J. Büttner. Proceeding of the IFCC Meeting on Reference Materials and Reference Systems co sponsored by WHO. Eur. J. Clin. Chem. Clin. Biochem., 33 (1995) 975-1022

[3] C. W. Weykamp, T. J. Penders, F. A. J. Muskiet, W. van der Slik. Effect of calibration on dispersion of glycohemoglobin values determined by 111 laboratories using 21 methods. Clin. Chem., 40 (1994) 138-144

[4] C. W. Weykamp, T. J. Penders, K. Miedema, F. A. J. Muskiet, W. van der Slik. Standardization of glycohemoglobin results and reference values in whole blood studied in 103 laboratories using 20 methods. Clin. Chem., 41 (1995) 82-86

[5] J.-O. Jeppsson, U. Kobold, J. Barr, A. Finke, W. Hoelzel, T. Hoshino, K. Miedema, A. Mosca, P. Mauri, R. Paroni, L. Thienpont, M. Umemoto, C. W. Weykamp. Approved IFCC reference method for the measurement of HbA1c in human blood. Clin. Chem. Lab. Med., 40 (2002) 78-89

[6] International Committee for Standardization in Haematology (ICSH). Recommendations for reference method for haemoglobinometry in human blood (ICSH Standard EP 6/2 1977) and specifications for international haemoglobincyanide reference preparation (ICSH Standard EP 6/3 1977). J. Clin. Pathol. 31 (1978/139-143).

[7] T. Linsinger, J. Pauwels, A. Lamberty, H. Schimmel, A. M. H. van der Veen, L. Siekmann. Estimating the uncertainty of stability for matrix CRMs. Fresenius J. Anal. Chem., 370 (2001) 183-188

[8] T. Linsinger, J. Pauwels, A. M. H. van der Veen, H. Schimmel, A. Lamberty. Homogeneity and stability of reference materials. Accred. Qual. Assur., 6 (2001) 20-25

[9] A. Finke, U. Kobold, W. Hoelzel, C. W. Weykamp, K. Miedema, J.-O. Jeppsson. Preparation of a candidate primary reference material for the international standardisation of HbA1c determinations. Clin. Chem. Lab. Med., 36 (1998) 299-308

[10] J. Pauwels, A. Lamberty, H. Schimmel. The determination of the uncertainty of reference materials certified by laboratory intercomparison. Accred. Qual. Assur., 3 (1998) 180-184

[11] ISO-IEC-BIPM-IFCC-IUPAC-IUPAP-OIML. Guide to the Expression of Uncertainty in Measurement (GUM), ISO Geneva, Switzerland (ISBN 92-67-10188-9), 1995

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ANNEX 1: ADDITIONAL CHARACTERISATION MEASUREMENTS

Evaluation of IRMM/IFCC-466 using Tosoh HPLC set to quantify HbA1c.

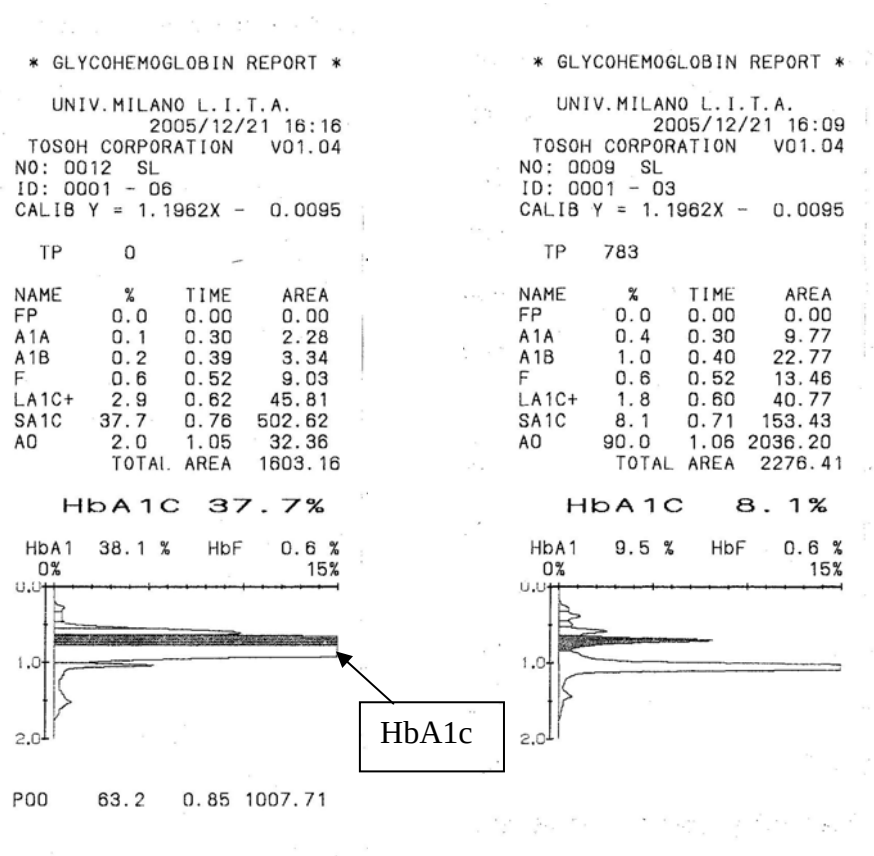

Figure I. Example for a single run c*hromatogram obtained by Tosoh HPLC (HbA1c program). Whole tetrameric haemoglobins are separated and followed at 415 nm. Peak "LA1C+" is an unknown Hb adduct eluted at the same retention time as the labile form of HbA1c. The peak corresponding to HbA1c is split for IRMM/IFCC-466 (figure on the left) but not for the fresh haemolysate (figure on the right).*

Evaluation of IRMM/IFCC-466 using Tosoh HPLC set to quantify HbA0.

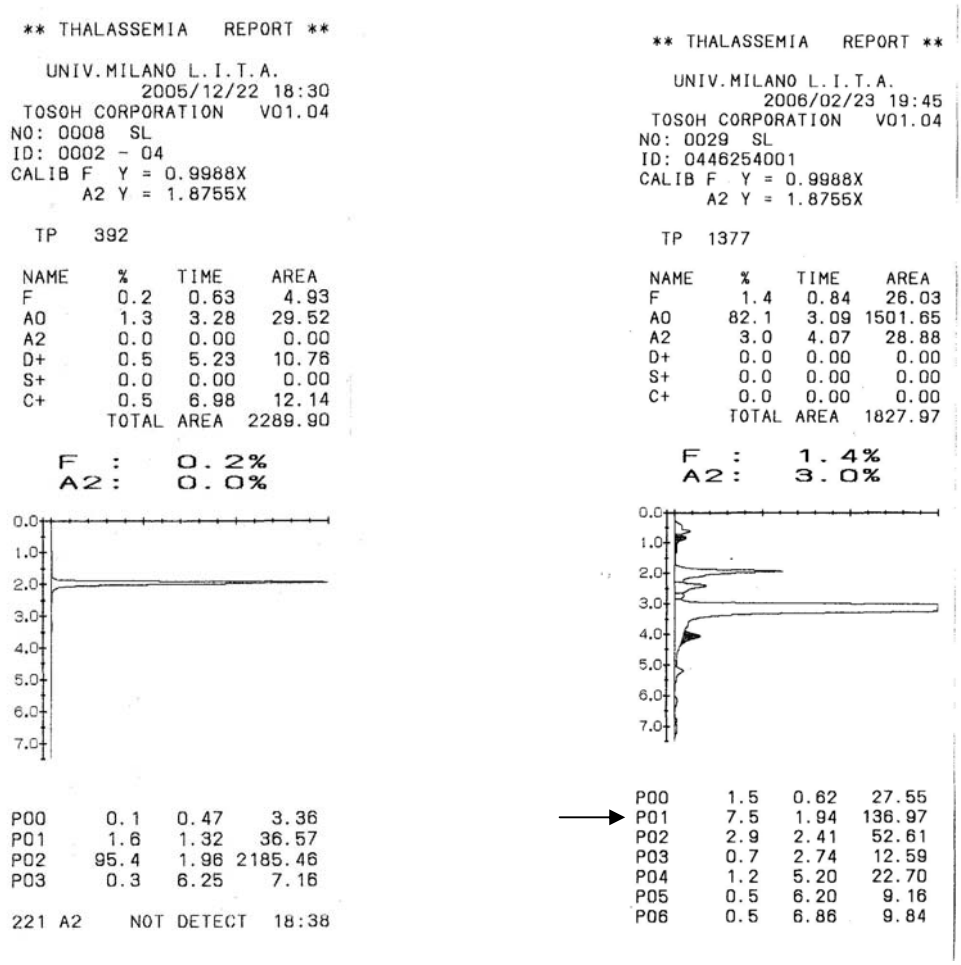

Figure II. *Example for a single run chromatogram obtained by Tosoh HPLC (HbA2 program). Left) IRMM/IFCC-466, right) fresh haemolysate. Whole tetrameric haemoglobins are separated and followed at 415 nm.*

Evaluation of IRMM/IFCC-466 using CEofix A1c Analis kit

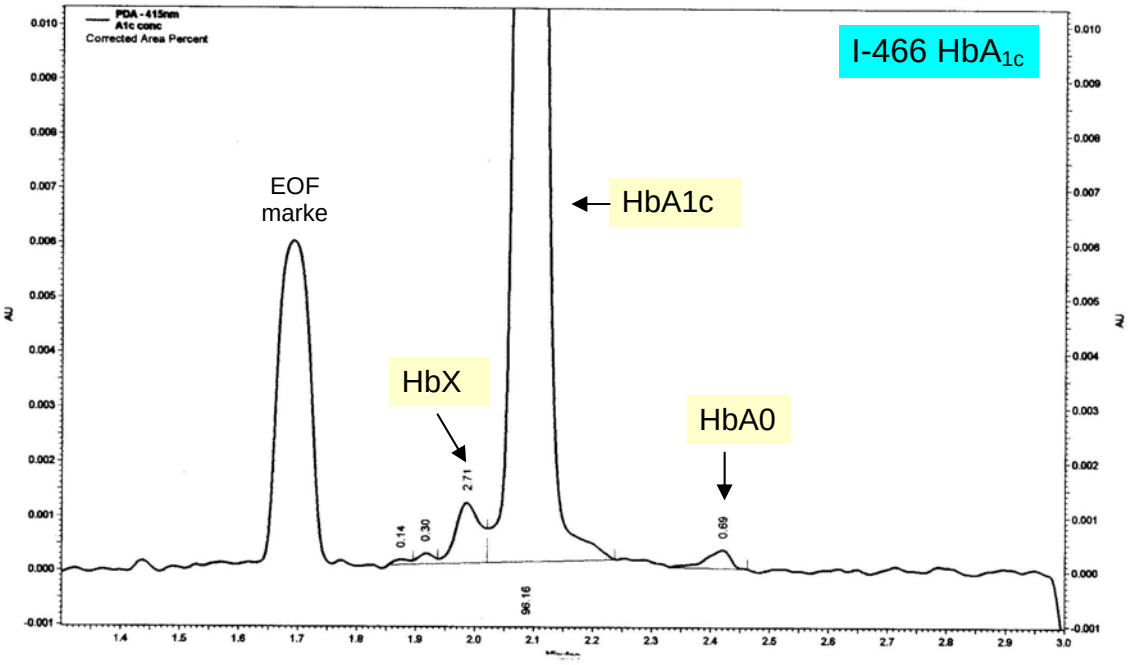

Figure III. *Capillary electrophoresis for IRMM/IFCC-466. Whole tetrameric haemoglobins are separated and followed at 415 nm (HbX corresponds to an unknown form of Hb).*

Absorption spectra for IRMM/IFCC-466 and fresh haemolysate showing the region (400-800 nm) corresponding to the haeme group.

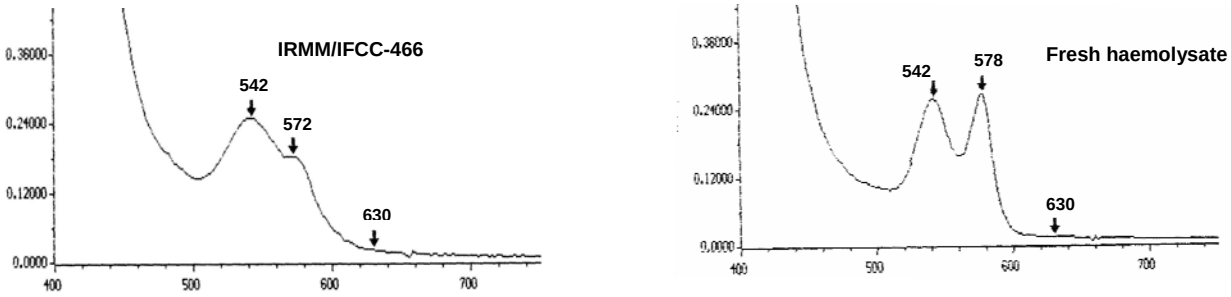

Figure IV. *Absorption spectra of IRMM/IFCC-466 and fresh haemolysated showing the differences in oxidation states.*

European Commission

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Abstract

This report describes the preparation, homogeneity, stability and certification studies as well as the purity evaluation of highly purified HbA1c (IRMM/IFCC-466) from human blood. HbA1c is defined as the stable adduct from glucose and the N-terminal amino group of the β-chain of haemoglobin A0 that is beta-N-(1-deoxyfructos-1-yl) haemoglobin [1]. A description of the analytical methods used in each of these studies is included. All relevant data from the homogeneity and stability studies as well as those for the characterisation measurements are presented.
The certified value is:

	Certified value[1)] Amount-of-substance fraction [mmol/mol]	Uncertainty[2)] [mmol/mol]
HbA1c/(HbA1c + HbA0)[3)]	934	22

[1)] The certified value was calculated as the average of the results for the amount-of-substance fraction of HbA0 versus HbA0 plus HbA1c of three accepted datasets and converted into amount-of-substance fraction HbA1c (1000 mmol/mol – HbA0 mmol/mol). Measurements were carried out using the IFCC reference measurement procedure [2] and further confirmed by other methods. The certified value, expressed as mmol HbA1c per mol HbA1c plus HbA0, is traceable to the SI.
[2)] The certified uncertainty is the expanded uncertainty estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM), ISO, 1995. It is expressed with a coverage factor (k = 2), corresponding to a level of confidence of about 95 %.
[3)] HbA1c is defined as the beta-N-(1-deoxyfructos-1-yl) haemoglobin. HbA0 is defined as the non-glycated haemoglobin.

The impurity consisting of HbA0 in IRMM/IFCC-466 was found to be 66 mmol/mol ± 22 mmol/mol (expressed as mmol HbA0 per mol HbA1c plus HbA0).
The total haemoglobin mass fraction in the material was also determined and the corresponding value (26.2 ± 0.9 mg Hb/g of solution) is provided as additional material information.
The intended use of this certified reference material is the calibration of the IFCC reference measurement procedure and other analogous methods based on the quantification of the N-terminal hexapeptide that includes the stable glycation.
The commutability of the material with routine *in vitro* diagnostic devices has not been assessed. Thus, prior to be used as calibrant for routine *in vitro* diagnostic devices, the commutability of IRMM/IFCC-466 should be investigated by the user.

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